

Chlorination of lignin by ubiquitous fungi has a likely role in global organochlorine production

Patricia Ortiz-Bermúdez*, Kolby C. Hirth†, Ewald Srebotnik‡, and Kenneth E. Hammel*†§

*Department of Bacteriology, University of Wisconsin, Madison, WI 53706; †United States Department of Agriculture Forest Products Laboratory, Madison, WI 53726; and ‡Fakultät für Technische Chemie, Technische Universität Wien, A-1060 Vienna, Austria

Edited by Derek Muir, Environment Canada, Burlington, ON, Canada, and accepted by the Editorial Board January 10, 2007 (received for review November 13, 2006)

Soils and decayed plant litter contain significant quantities of chlorinated aromatic polymers that have a natural but largely unknown origin. We used cupric oxide ligninolysis coupled with gas chromatography/mass spectrometry to show that *Curvularia inaequalis*, a widely distributed litter ascomycete, chlorinated the aromatic rings of lignin in wood that it was degrading. In aspen wood decayed for 24 weeks, two chlorolignin fragments, 5-chlorovanillin and 2-chlorosyringaldehyde, were each found at $\approx 10 \mu\text{g/g}$ of wood (dry weight). These levels resemble those of similar structures generally found in unpolluted environmental samples. Fractionation of the extractable proteins followed by tandem mass spectrometric analysis showed that the colonized wood contained a previously described *C. inaequalis* chloroperoxidase that very likely catalyzed lignin chlorination. Chlorolignin produced by this route and humus derived from it are probably significant components of the global chlorine cycle because chloroperoxidase-producing fungi are ubiquitous in decaying lignocellulose and lignin is the earth's most abundant aromatic substance.

chloroperoxidase | humus | hypochlorous acid | Pleosporales | soil

Chlorinated organic compounds occur naturally in soils, sediments, and dead plant biomass and constitute a major reservoir in biogeochemical chlorine cycling (1–3). Given the magnitude of the cycle and the toxicity of many chlorinated chemicals, it would be useful to know more about the mechanisms that produce natural organochlorine. Recent x-ray spectroscopic work on extensively decayed plant litter has revealed that most of its chlorine is contained in high-molecular weight aromatic structures (4). Some of these residues are probably formed when various naturally occurring low-molecular weight chloroaromatics undergo humification (3). However, it has also been noted that this high-molecular weight material contains chlorinated guaiacyl (4-hydroxy-3-methoxy-substituted) and syringyl (4-hydroxy-3,5-dimethoxy-substituted) aromatic rings typical of lignin (5–7). Lignin, a structural polymer of vascular plants and contributor to humus formation, is the most abundant aromatic substance on earth and the second largest constituent of terrestrial biomass after plant polysaccharides (8). A large quantity of organochlorine would likely result if a widespread mechanism exists to chlorinate it *in situ*.

One possibility is that lignin is directly chlorinated by some filamentous fungi when they degrade lignocellulose. A variety of plant pathogenic ascomycetes that also occur as litter saprophytes have been reported to secrete chloroperoxidases (CPOs) on defined culture media (9, 10), and uncharacterized CPO activity has also been detected in soils (11), which generally contain significant fungal biomass (12). Fungal CPOs are heme- or vanadium-containing enzymes that catalyze the H_2O_2 -dependent oxidation of Cl^- to hypochlorous acid (HOCl) or a similarly reactive chlorine electrophile, and as a result they chlorinate lignin and related aromatic structures *in vitro* (13–16). It remains to be seen whether fungal CPOs are expressed in lignocellulose or chlorinate it *in vivo*, but the hypothesis is attractive because the mycelial growth habit of lignocellulolytic

fungi places secreted enzymes in very close contact with targeted plant cell wall polymers. Because HOCl is too reactive to diffuse over long distances (17, 18), this juxtaposition would increase the likelihood that lignin is chlorinated.

Litter-inhabiting ascomycetes in the Pleosporales (19) stand out as candidates for a test of the hypothesis that fungi chlorinate lignin. They are widely distributed on woody and herbaceous debris in diverse environments, where some of them contribute to lignocellulose breakdown (20). Many members of the order produce haloperoxidase activity on laboratory media (10, 21), and in some cases these enzymes have been identified as vanadium CPOs (vCPOs) (22, 23). In others, enzymological work has not yet been done, but the presence of DNA sequences with high similarity to known vCPO genes suggests that these enzymes may be produced (e.g., see National Center for Biotechnology Information accession no. EAT82270 for *Phaeosphaeria nodorum*). Thus, vCPOs could have a major role in global biochlorinations, as first proposed some years ago by Wever and his colleagues (10, 23). For our experiments, we selected *Curvularia inaequalis*, a vCPO-producing, cosmopolitan species in the Pleosporales that has been found on diverse plants, in maritime sand dune soil, and on wood in brackish water (20, 24–26).

Results and Discussion

Colonization of Wood. We grew *C. inaequalis* on wafers of aspen and spruce, and also on aspen meal, in the presence of auxiliary nutrients that wood-decay ascomycetes generally require to degrade lignocellulose efficiently (27, 28). Because the biodegradative capabilities of this species have not previously been assessed, we monitored the extent of decay in colonized wafers at intervals by transmission electron microscopy and by determining losses in sample dry weight. *C. inaequalis* aggressively decayed the aspen, an angiosperm wood, by eroding the secondary cell wall from the lumen inward (Fig. 1), causing $18 \pm 3\%$ weight loss in 12 weeks and $33 \pm 3\%$ weight loss in 24 weeks ($n = 4$). An erosive decay can occur only if all components of the wood are removed, including lignin, and therefore *C. inaequalis* qualifies as a lignocellulolytic litter ascomycete. This result indicates that the fungus can probably colonize angiosperm lignocellulose for an extended time under natural conditions, and it is consequently a suitable candidate to test for chlorination of lignin.

An examination of the spruce showed that *C. inaequalis* colonized the cell lumens extensively but without causing decay (data not shown). Therefore, although some Pleosporales can

Author contributions: P.O.-B. and K.E.H. designed research; P.O.-B., K.C.H., and E.S. performed research; P.O.-B., K.C.H., E.S., and K.E.H. analyzed data; and K.E.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission. D.M. is a guest editor invited by the Editorial Board.

Abbreviations: CPO, chloroperoxidase; dd, distilled, deionized; vCPO, vanadium chloroperoxidase.

§To whom correspondence should be addressed. E-mail: kehammel@wisc.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0610074104/DC1.

© 2007 by The National Academy of Sciences of the USA

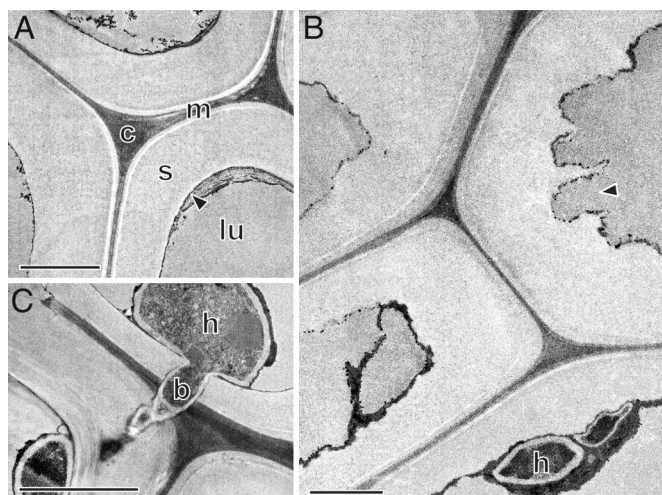


Fig. 1. Transmission electron microscopic views of aspen wood in transverse section. (A) Cells of sound aspen are shown with the secondary layer (s) that constitutes the bulk of the woody material, the highly lignified middle lamella (m), and the cell corner (c) regions all in their undegraded state. Note that the lumen (lu) surface of the cells (arrowhead) is free of notches and serrations. (B) Serrated edges and deep notches (arrowhead) all along the lumen surface were evident in the majority of aspen cells colonized by hyphae (h) of *C. inaequalis* for 24 weeks. Thus, the fungus degraded aspen by removing all components, including lignin, progressively inward from the lumen surface. Cavities within the wood cell wall (which are typical of many wood-decay ascomycetes) were not observed with *C. inaequalis*. (C) A narrow-bore hypha (b) of *C. inaequalis* is shown extending from a normal hypha (h) and penetrating all wood cell wall layers, including the middle lamella. Such bore hyphae are thought to facilitate wood colonization and were observed frequently. In this particular case the wood cells were still not eroded, which indicates that bore hyphae form at the initial stage of degradation. (Scale bars, 5 μm .)

degrade gymnosperm wood (29), it is apparently not a growth substrate for *C. inaequalis* in natural settings. Nevertheless, we included spruce-grown cultures as a control to determine whether any chlorinated structures we might find were consistent with the lignin composition in the colonized wood; angiosperm lignin generally contains significant levels of both syringyl and guaiacyl structures, whereas gymnosperm lignin is composed almost entirely of guaiacyl units (8).

Chlorination of Wood. To analyze the colonized wood samples for chlorolignin, we oxidized them with alkaline CuO, separated the resulting lignin fragments by preparative HPLC, and analyzed the eluates by GC coupled with electron ionization MS. Alkaline CuO cleaves native lignin in 20–40% yield to give soluble products that are dominated by benzaldehydes diagnostic for the type of lignin analyzed—guaiacyl structures yield vanillin, whereas syringyl structures yield syringaldehyde. If a chlorine electrophile has reacted with the lignin, chlorinated derivatives of these benzaldehydes occur as additional products (30) and are discernible from their characteristic $^{35}\text{Cl}/^{37}\text{Cl}$ isotopic signatures in MS experiments.

The results showed that the colonized spruce yielded 5-chlorovanillin, that the colonized aspen yielded both 5-chlorovanillin and 2-chlorosyringaldehyde, and that chlorinated benzaldehydes were undetectable in uncolonized wood (Table 1 and Fig. 2). Because the pattern of chlorination agreed with the type of lignin in each wood, it is evident that the chlorinated aromatics did not originate as fungal metabolites, but rather came from the lignins. Furthermore, the yields of chlorinated products from wood colonized for 18 or 24 weeks were on the same order of magnitude as those of similar products obtained from typical

Table 1. Levels of chlorinated lignin fragments found by GC/MS analysis after CuO oxidation of wood colonized by *C. inaequalis*

Culture conditions	Concentration of product, $\mu\text{g/g}$ wood (dry wt) \pm SD of the sample	
	5-Chlorovanillin	2-Chlorosyringaldehyde
Inoculated spruce wafers (18 wk, $n = 5$)	17.3 ± 6.9	ND*
Uninoculated spruce ($n = 2$)	ND	ND
Inoculated aspen wafers (12 wk, $n = 3$)	2.2 ± 1.6	2.5 ± 1.4
Inoculated aspen wafers (24 wk, $n = 4$)	9.9 ± 4.5	13.8 ± 5.8
Inoculated aspen meal (6 wk, $n = 5$)	2.9 ± 0.9	6.9 ± 2.9
Uninoculated aspen ($n = 3$)	ND	ND

*ND, not detected. Detection limit = 0.5 $\mu\text{g/g}$.

field samples. For example, decaying spruce wood and fulvic acids from unpolluted lake water were reported to yield $\approx 40 \mu\text{g/g}$ and $9 \mu\text{g/g}$, respectively, of chlorinated guaiacyl structures (5, 7).

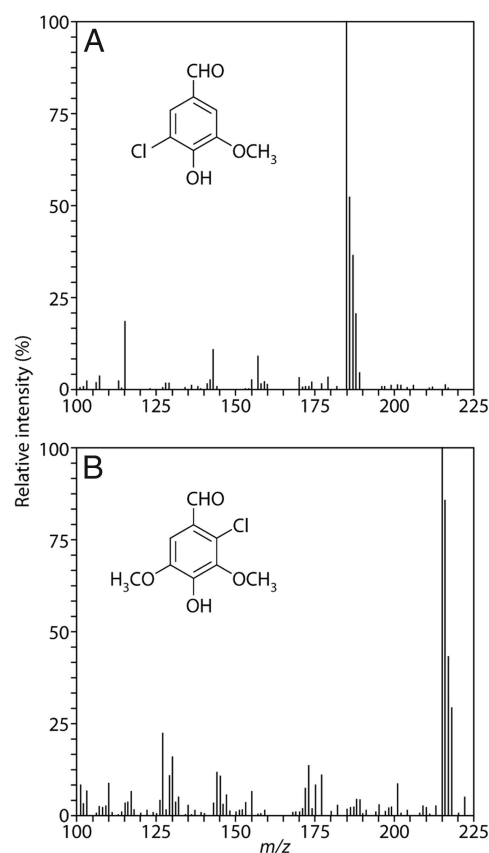


Fig. 2. Mass spectra of chlorinated lignin fragments found by GC/MS analysis after CuO oxidation of wood colonized by *C. inaequalis*. (A) 5-Chlorovanillin from a spruce wafer colonized by *C. inaequalis* for 18 weeks, showing diagnostic ions with m/z 188 ($M^+ + 2$), 187 ($M^+ + 2 - 1$), 186 (M^+), and 185 ($M^+ - 1$). The level of 5-chlorovanillin in this wood wafer was 15.1 $\mu\text{g/g}$ (dry weight). (B) 2-Chlorosyringaldehyde from an aspen wafer colonized for 24 weeks, showing diagnostic ions with m/z 218 ($M^+ + 2$), 217 ($M^+ + 2 - 1$), 216 (M^+), and 215 ($M^+ - 1$). The level of 2-chlorosyringaldehyde in this wafer was 10.1 $\mu\text{g/g}$ (dry weight).

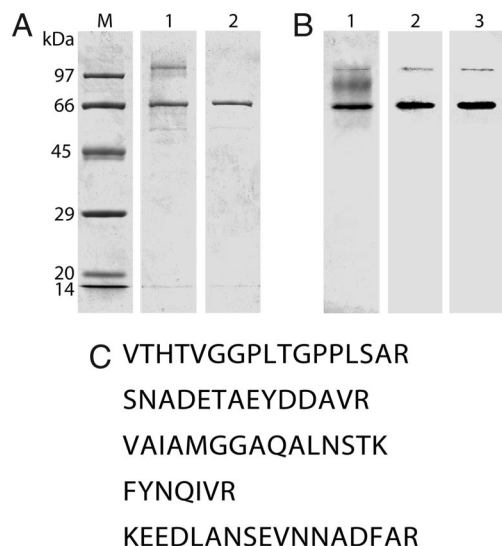


Fig. 3. Detection of a *C. inaequalis* CPO in wood colonized by the fungus. (A) Denaturing SDS/polyacrylamide gels stained for protein, showing molecular mass markers (lane M), the enzyme purified from colonized wood (lane 1), and the enzyme purified from glucose-grown cultures as described previously (23) (lane 2). (B) Nondenaturing SDS/polyacrylamide gels, showing the enzyme purified from wood and stained for protein (lane 1), the enzyme purified from wood and stained for haloperoxidase activity (lane 2), and the enzyme purified from glucose-grown cultures and stained for haloperoxidase activity (lane 3). Lane 3 is from a different gel than the one containing lanes 1 and 2. The minor, slower-migrating haloperoxidase activity seen in lanes 2 and 3 is an aggregate of the CPO reported earlier (23). The activity stain depends on the haloperoxidase-catalyzed bromination of *o*-dianisidine in the presence of H₂O₂ and Br⁻. In our gels, no staining occurred when either H₂O₂ or Br⁻ was omitted (data not shown). (C) Peptide sequences identified in a tryptic digest of the major 67-kDa band from a denaturing gel.

6-Chlorovanillin was not detectable in any sample. Because the 3-methoxyl substituent in nonphenolic guaiacyl lignin structures is expected to direct a significant fraction of attacking chlorine electrophiles *para* to the 6-position (31), it appears that relatively few nonphenolic structures were chlorinated. Our detection of only 5-chlorovanillin in the samples likely indicates selective chlorination of phenolic guaiacyl units because the aromatic hydroxyl group in these structures activates the adjacent 5-position toward electrophilic attack (31). This result is unsurprising; even though the frequency of phenolic units in lignin is only on the order of 10–20% (32), the rate constant for addition of HOCl to a phenol is several hundredfold higher than for addition to a methoxybenzene at pH 6.5, the acidity of our cultures (17, 18).

Production of CPO in Wood. Haloperoxidase activity was present in all wood wafer and wood meal cultures within 3–6 weeks and persisted throughout the experiments, as shown by the ability of colonized wood samples to brominate the dye phenol red rapidly in the presence of exogenous H_2O_2 and NaBr. The product, bromophenol blue, exhibits a purple color that makes this semiquantitative assay sensitive for haloperoxidase detection (21). Using this method to track the activity, we purified the haloperoxidase from extracts of 6-week aspen meal cultures by ion exchange, hydrophobic interaction, and gel-permeation chromatography. The activity ran as a single peak in all steps, yielding a preparation that was $\approx 80\%$ pure, with the major component exhibiting a molecular mass of 67 kDa as determined by denaturing SDS/PAGE (Fig. 3A). Activity staining of gels subjected to SDS/PAGE under nondenaturing conditions

showed that the haloperoxidase was the principal component in the final, purified protein sample (Fig. 3*B*).

The 67-kDa band on a denaturing gel of the haloperoxidase from colonized wood was excised, and a tryptic digest of it was analyzed by tandem mass spectrometry with a search of the resulting data against the nonredundant National Center for Biotechnology Information database. Five tryptic peptides with unambiguous sequence tags gave unique matches, with 11% coverage, to the 67-kDa *C. inaequalis* vCPO purified earlier from glucose-grown cultures [Fig. 3C and [supporting information \(SI\) Fig. 4](#)] (33). Therefore, this CPO is expressed by *C. inaequalis* on wood and very likely catalyzed the lignin chlorinations we observed. Of course, the data do not exclude additional chlorination mechanisms, and no genetic transformation system is yet available to perform a diagnostic *cpo* gene disruption in *C. inaequalis*.

Conclusions

In a previous report (15), we showed that purified fungal CPOs chlorinate lignin structures *in vitro*, but it remained uncertain whether fungi actually express these enzymes and chlorinate lignin while growing on their natural substrates. This result is not easy to demonstrate in field samples of lignocellulose, which lack a known history and contain mixed microbial populations, but the experiment is straightforward with axenic cultures. By using this approach with a widespread fungus, we have now provided evidence that fungal, CPO-mediated chlorination of lignin has a role in natural organochlorine production.

For several reasons, we surmise that this process is most rapid in maritime ecosystems such as salt marshes and mangrove swamps. First, ambient Cl^- concentrations in these environments generally exceed 100 mM, the K_m of a typical vCPO for Cl^- at pH 8, the acidity of seawater (23, 34). Second, the rate of HOCl addition to phenolic lignin structures is expected to be rapid (≈ 100 liters per mol per s) at pH 8 (17). Finally, species in the Pleosporales are abundant on plant detritus in these environments (35–37). Our culture conditions for *C. inaequalis* on wood at pH 6.5 and $[\text{Cl}^-] = 20$ mM reflect the less saline maritime settings where this fungus has been found (25, 26).

At the other extreme are temperate forest soils, with acidities around pH 4 and Cl^- concentrations <1 mM. Under these conditions, chlorination is probably slower, even though vCPOs have K_m values of 1 mM or lower for Cl^- at pH 4 (23) because rate constants for the reaction between HOCl and aromatic structures are only ≈ 1 liter per mol per s at this acidity (17, 18). On the other hand, phenolic and nonphenolic lignin structures are probably chlorinated at similar rates in acidic environments because it is the ionization of phenols with increasing pH that makes them so reactive with HOCl (17). If phenolic lignin structures are chlorinated less preferentially under acidic conditions, then 6-chlorinated guaiacyl products should occur as well as 5-chlorinated ones in plant residues from such environments. This result has been obtained in one analysis of surface water from a peat bog (6).

In proposing that the fungal chlorination of lignin contributes to global organochlorine production, we recognize that other processes also have a role. This complexity is evident from the observation that depolymerizates of high-molecular weight material from pristine environments generally contain greater quantities of nonmethoxylated 4-hydroxy-substituted chloroaromatics than of methoxylated ones (5–7). Although some of these products probably originate from the CPO-mediated chlorination of nonmethoxylated lignin structures and associated coumaryl esters, which occur at significant levels in grasses (8), they appear too abundant to come only from lignin. It is likely that most of the nonmethoxylated chloroaromatics in soils and plant litter arise from the demethylation and subsequent humification

of 4-methoxylated chloroaromatic natural products, which many wood-decay basidiomycetes produce at high levels (38, 39).

Our work brings up additional questions for future research. First, CPOs have been shown to cleave lignin *in vitro* (15) and might thus contribute to ligninolysis *in vivo* as proposed earlier (10). The results we obtained with *C. inaequalis* on spruce indicate that chlorine electrophiles were not produced in sufficient quantity to cause wood decay by themselves, but it is possible that CPO is one component of a broader biodegradative arsenal that litter-decay Pleosporales use to degrade plant cell wall material. Second, our data show that wood cell wall erosion and lignin chlorination by *C. inaequalis* proceeded simultaneously, which suggests that some of the chlorolignin that was formed was also degraded. Thus, litter-decay ascomycetes, together with ligninolytic basidiomycetes (38, 40), may initiate the recycling of recalcitrant soil organochlorine by depolymerizing it. The subsequent mineralization of the resulting low-molecular weight organochlorine is, of course, accomplished by a wide variety of bacteria and fungi (38, 41–43).

Materials and Methods

Reagents and Organism. 5-Chlorovanillin, 6-chlorovanillin, and 2-chlorosyringaldehyde were obtained from Helix Biotech (Richmond, BC, Canada). 4-Methoxycinnamaldehyde was from the chemical collection of the U.S. Department of Agriculture Forest Products Laboratory, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). Solvents were HPLC grade, except for the acetone used in GC/MS work, which was pesticide residue analysis grade. *C. inaequalis* (ATCC 10713) was maintained on malt agar plates, which were incubated at 24°C and then stored at 4°C.

Wood Wafer Cultures. *C. inaequalis* was grown on 3- × 10- × 30-mm wafers of aspen (*Populus tremuloides*) or spruce (*Picea glauca*) that had been cut with the large face perpendicular to the grain. The blocks were autoclaved twice and placed on nylon mesh over 50 ml of previously inoculated nutrient agar in Petri dishes (150-mm diameter). An agar plug of inoculum was placed on top of each block, and the plates were incubated at 24°C for 12, 18, or 24 weeks.

The nutrient agar was a modification of the CFM medium used earlier (21), containing 24.0 g/liter potato dextrose broth, 3.0 g/liter yeast extract, 20 mM NaCl, 100 μM NaVO₃, and the trace element solution originally specified. In natural settings, soil provides lignocellulolytic ascomycetes with the auxiliary nutrients necessary for efficient wood decay. However, because soils already contain chlorinated aromatic residues that might have entered the wood, we used nutrient agar as a synthetic substitute.

Aspen Meal Cultures. Two agar plugs of *C. inaequalis* were inoculated into each of 10 250-ml Erlenmeyer flasks containing 50 ml of FA-1 germination medium (21). The flasks were rotary-shaken at 250 rpm and 24°C for 5 days, after which the mycelia were harvested, pooled, washed twice with sterile, distilled, deionized H₂O (ddH₂O), and then homogenized in an electric blender. The homogenate (100 ml) was added to 200 ml of sterile CFM medium, which was prepared as described above except that the agar was omitted, and the amounts of potato dextrose broth and yeast extract were decreased to 5.00 and 1.25 g/liter, respectively. For each culture, 6.0 ml of this inoculum was added to a 250-ml Erlenmeyer flask containing 2 g of aspen wood that had previously been milled to pass a 2-mm sieve, moistened with 10 ml of ddH₂O, and autoclaved twice. The cultures were covered with aluminum foil and grown statically at 24°C for 6 weeks. At this time, the pH of several cultures was checked and found to be 6.5.

Assessment of Wood Decay and Haloperoxidase Activity in Cultures.

The extents of decay were monitored in the colonized wood wafers by determining changes in sample dry weight and by transmission electron microscopy. For the latter procedure, specimens (1 × 1 × 3 mm) were cut from sound and degraded wafers and fixed in 12 mM phosphate buffer (pH 7.3) containing 2% glutaraldehyde for 16 h at 4°C, postfixed in 1% OsO₄ for 2 h at ambient temperature, stained in 2% uranyl acetate for 16 h at ambient temperature, dehydrated through a graded acetone series, and finally embedded in Spurr's low-viscosity resin (Ted Pella, Redding, CA). Ultrathin sections were examined in a JEOL 100CX electron microscope (JEOL, Tokyo, Japan) at 100 kV.

Extracellular haloperoxidase production by the cultures was detected by taking small portions from colonized wood meal or cut pieces from colonized wood wafers and assaying their ability to convert phenol red to bromophenol blue as described earlier (21).

CuO Oxidations. Wood samples were ground to 60 mesh in a Wiley mill, put in cellulose thimbles (Whatman, Florham Park, NJ), and extracted with acetone/water (10:1 vol/vol) in a Soxhlet apparatus for 48 h as described previously (30). The samples were then air-dried overnight and stored over P₂O₅ for several days under vacuum. Dried samples (200 mg) were weighed into 20-ml glass crimp-top vials, to each of which 1.0 g of CuO and 7.0 ml of 2 M NaOH were added. The reaction vials were sealed with inert septa under N₂ and placed inside a stainless steel bomb, which was also sealed under N₂. The apparatus was heated to 170°C, and the vials were mechanically rotated at 60 rpm for 2 h, after which the apparatus was cooled, and the samples were taken out.

Each sample was centrifuged for 30 min at 47,600 × g, and the supernatant fraction containing lignin-derived aromatic fragments was saved. The pellet was resuspended and centrifuged again in 10 ml of ddH₂O, after which the resulting supernatant fraction was combined with the first one. The pooled sample was stirred and slowly acidified to pH 2.0 with 20% HCl, left at room temperature for 1 h, and then centrifuged as above for 30 min. The supernatant fraction was saved, and the pellet was resuspended in 12 mM HCl for a second centrifugation. The two supernatant fractions were then combined and vacuum-filtered through 0.45-μm pore nylon filters (Millipore, Billerica, MA), after which 10.0 μg of 4-methoxycinnamaldehyde was added to each sample as an internal standard. The soluble oxidation products were then adsorbed to solid-phase extraction columns (Hi-Load C18, 1,000 mg, Alltech, Nicholasville, KY) that had been washed with methanol and then preequilibrated with aqueous 12 mM HCl. Products were eluted with 4 ml of ethyl acetate. The solvent was then evaporated, and the samples were redissolved in acetonitrile/ddH₂O, 93:7.

Chlorinated lignin oxidation products were separated from more polar nonchlorinated products (chiefly vanillin, acetovanillone, syringaldehyde, and acetosyringone) by preparative reverse-phase HPLC on a Vydac 201TP1010 C18 column (210 mm long × 10-mm diameter, Alltech). The column was eluted at 5 ml/min and ambient temperature with a 25-min 10–40% linear gradient of acetonitrile in ddH₂O. The compounds destined for GC/MS analysis had the following retention times: 5-chlorovanillin, 17.7 min; 6-chlorovanillin, 19.4 min; 2-chlorosyringaldehyde, 19.5 min; 4-methoxycinnamaldehyde, 23.2 min. Accordingly, for each sample the fraction eluting between 15 and 25 min was collected and concentrated by solid-phase extraction as described above. The purified extracts were evaporated, redissolved in 300 μl of acetone, and filtered through 0.45-μm pore filters (Millipore).

GC/MS Analysis. All measurements were carried out with a Varian 4000 ion trap mass spectrometer (Walnut Creek, CA) coupled to a Varian 3800 gas chromatograph that was

equipped with a CTC Analytics Combi-Pal autosampler (Zwingen, Switzerland). A Siltek-passivated single-gooseneck liner (Restek, Bellefonte, PA) and a Supelco Wax10 column (30 m long \times 0.25-mm inner diameter, 0.25- μ m film thickness, Sigma-Aldrich) were used. Splitless injections (1 μ l) were performed. Operating conditions were as follows: injector temperature, 220°C; carrier gas, helium at 1.0 ml/min; oven temperature program, 150–275°C at 45°C/min and then held for 5 min. The mass spectrometer was operated in positive electron ionization mode (70 eV) over a range of m/z 100–250 at the following temperatures: source, 170°C; manifold, 40°C; transfer line, 250°C. The instrument was operated at 0.42 s per scan with averaging of every three scans, which yielded more than eight points per peak.

Analyte identification was based on comparisons of mass spectra and retention times with those of authentic standards. Retention times were as follows: 4-methoxycinnamaldehyde, 4.2 min; 6-chlorovanillin, 5.2 min; 5-chlorovanillin, 5.9 min; 2-chlorosyringaldehyde, 6.8 min. To quantify the monochlorinated benzaldehydes, we monitored their molecular ions (M^+ and $M^+ + 2$) and the ions attributable to the loss of the aldehyde proton ($M^+ - 1$ and $M^+ + 2 - 1$) and compared the integrated results against external linear calibration curves ($r^2 \geq 0.998$) of at least 9 points that bracketed the samples and spanned the range from 0.13 to 15 ng/ μ l. 4-Methoxycinnamaldehyde was used as an internal standard to normalize the sample volumes. Two injections of acetone were chromatographed between every sample and standard, and no carryover was detected. The limit of detection for the chlorinated benzaldehydes in wood samples was 0.5 μ g/g of wood (dry weight), based on the requirements that the signal/noise ratio had to be ≥ 3 and that the characteristic $^{35}\text{Cl}/^{37}\text{Cl}$ isotopic pattern of the monochlorinated analytes had to be unambiguously apparent relative to background ions in the spectra (SI Fig. 5).

Protein Extraction from Wood. All steps were done at 4°C. Sixteen 6-week-old cultures of *C. inaequalis* growing on aspen meal were pooled in 300 ml of extraction buffer (50 mM Tris-HCl/0.5 M NaCl/2% polyvinylpyrrolidone at pH 7.2), and rotary-shaken (150 rpm) for 45 min. The slurry was then centrifuged (15 min, 23,000 \times g), and the supernatant fraction was filtered first through a glass fiber filter and then through a 0.45- μ m pore nylon filter (Millipore). The pellet was resuspended in fresh extraction buffer and extracted again by the same procedure, after which the pooled filtrates (\approx 600 ml) were concentrated to a volume of \approx 5 ml in an ultrafiltration cell (Millipore) fitted with a 10-kDa cutoff polyethersulfone membrane, and then dialyzed by repeated ultrafiltration in 50 mM potassium phosphate (pH 7.0), using the same apparatus.

CPO Purification. All steps were done at 4°C unless otherwise stated. The dialyzed and concentrated sample was loaded onto a 50-ml DEAE-Sepharcel ion-exchange column (Sigma-Aldrich) that had

been equilibrated beforehand with 50 mM potassium phosphate (pH 7.0). Proteins were eluted first with a 250-ml wash of 50 mM potassium phosphate (pH 7.0) and then with 250 ml each of 0.1, 0.2, 0.4, 0.6, and 0.8 M NaCl in the same buffer. The haloperoxidase activity eluted in the 0.4 M and 0.6 M NaCl fractions, which were concentrated to 5 ml by ultrafiltration as described above and then adjusted to 3 M NaCl. The sample was then loaded onto a 10-ml phenyl-Sepharose FF 6 hydrophobic interaction column (Sigma-Aldrich) that had been preequilibrated with 3 M NaCl in 50 mM potassium phosphate (pH 7.0). Proteins were eluted first with a 50-ml wash of 50 mM potassium phosphate (pH 7.0) containing 3 M NaCl and then with 50 ml of the same buffer without NaCl. The haloperoxidase activity eluted in the initial 3 M NaCl wash and was concentrated to a volume of 0.5 ml by centrifugation through a 10-kDa cutoff polyethersulfone membrane (Millipore). The sample was then subjected to gel-permeation chromatography on a medium pressure liquid chromatography system (FPLC; Amersham, Piscataway, NJ) fitted with a Superdex 200 column (30 cm long by 1-cm diameter, Amersham). The column was run at 0.2 ml/min in 20 mM piperazine HCl (pH 5.4) at room temperature. Fractions (0.5 ml) were collected, put on ice immediately, and assayed for haloperoxidase activity. The peak fractions (5 ml) were pooled and loaded directly onto a 1-ml Mono Q 5/50 GL ion-exchange column (Amersham) connected to the FPLC system. The column had been equilibrated beforehand with 20 mM piperazine HCl (pH 5.4) and was operated at 1 ml/min and room temperature. Proteins were eluted first with a 7-ml wash of 20 mM piperazine HCl (pH 5.4) and then with a 30-ml linear gradient of NaCl (0–0.5 M) in the same buffer. Fractions (1.0 ml) were collected, put on ice immediately, and assayed for haloperoxidase activity. The peak fractions (5 ml) eluted in the gradient and were concentrated and dialyzed against 10 mM Tris-HCl (pH 7.2).

Assays, Electrophoresis, and Activity Staining. Haloperoxidase activity was assessed semiquantitatively by using the phenol red colorimetric haloperoxidase activity assay as described above. Precast gels (10% acrylamide, in Tris-HCl, ReadyGel; Bio-Rad, Hercules, CA) loaded with 2–10 μ g of protein per lane were used for denaturing and nondenaturing SDS/PAGE. For nondenaturing gels, the protein samples were not boiled, and no 2-mercaptoethanol was used. Proteins in the gels were visualized by staining with Coomassie blue R-250. Haloperoxidase activity in nondenaturing SDS/gels was detected as described earlier (23) with an activity stain based on the H_2O_2 - and bromide-dependent bromination of *o*-dianisidine. Procedures for mass spectrometric protein identification are described in the SI Methods.

We thank C. J. K. Wang for advice on culture conditions, R. S. Reiner for assistance with the CuO oxidations of lignin, M. R. Suzuki for assistance with the CPO purification, G. Sabat for obtaining and interpreting the protein mass spectra, and J. Ralph for helpful discussions of the data. This work was supported by U.S. Department of Energy Grant DE-FG02-94ER20140 (to K.E.H.).

- Harper DB (2000) *Nat Prod Rep* 17:337–348.
- Öberg G (2002) *Appl Microbiol Biotechnol* 58:565–581.
- Winterton N (2000) *Green Chem* 2:173–225.
- Myneni SCB (2002) *Science* 295:1039–1041.
- Dahlman O, Morck R, Ljungquist P, Reimann A, Johansson C, Boren H, Grimvall A (1993) *Environ Sci Technol* 27:1616–1620.
- Dahlman O, Reimann A, Ljungquist P, Morck R, Johansson C, Boren H, Grimvall A (1994) *Water Sci Technol* 29:81–91.
- Flodin C, Johansson E, Boren H, Grimvall A, Dahlman O, Morck R (1997) *Environ Sci Technol* 31:2464–2468.
- Boerjan W, Ralph J, Baucher M (2003) *Annu Rev Plant Biol* 54:519–546.
- Hofrichter M, Ullrich R (2006) *Appl Microbiol Biotechnol* 71:276–288.
- Vollenbroek EGM, Simons LH, van Schijndel J, Barnett P, Balzar M, Dekker H, van der Linden C, Wever R (1995) *Biochem Soc Trans* 23:267–271.
- Asplund G, Christiansen JV, Grimvall A (1993) *Soil Biol Biochem* 25:41–46.
- Dix NJ, Webster J (1995) *Fungal Ecology* (Chapman & Hall, London), pp 172–183.
- Brown FS, Hager LP (1967) *J Am Chem Soc* 89:719–720.
- Niedan V, Pavasars I, Öberg G (2000) *Chemosphere* 41:779–785.
- Ortiz-Bermúdez P, Srebotnik E, Hammel KE (2003) *Appl Environ Microbiol* 69:5015–5018.
- Reina RG, Leri AC, Myneni SCB (2004) *Environ Sci Technol* 38:783–789.
- Gallard H, von Gunten U (2002) *Environ Sci Technol* 36:884–890.
- Pinkston KE, Sedlak DL (2004) *Environ Sci Technol* 38:4019–4025.
- Berbee ML (2001) *Physiol Mol Plant Pathol* 59:165–187.
- Ellis MB (1971) *Dematiaceous Hyphomycetes* (Commonwealth Mycol Inst, Kew, U.K.).
- Hunter-Cevera JC, Sotos L (1986) *Microb Ecol* 12:121–127.
- Barnett P, Hemrika W, Dekker HL, Muijsers AO, Renirie R, Wever R (1998) *J Biol Chem* 273:23381–23387.
- van Schijndel J, Vollenbroek EGM, Wever R (1993) *Biochim Biophys Acta* 1161:249–256.

24. Kim JC, Choi GJ, Kim HT, Kim HJ, Cho KY (2000) *Plant Dis* 84:684–688.
25. Moreau F (1941) *Rev Mycol* 6:49–94.
26. Shearer CA (1972) *Am J Bot* 59:961–969.
27. Worrall JJ, Anagnost SE, Wang CJK (1991) *Can J Microbiol* 37:869–874.
28. Worrall JJ, Anagnost SE, Zabel RA (1997) *Mycologia* 89:199–219.
29. Zabel RA, Wang CJK, Anagnost SE (1991) *Wood Fiber Sci* 23:220–237.
30. Chen C-L (1992) in *Methods in Lignin Chemistry*, eds Lin SY, Dence CW (Springer, Berlin), pp 301–321.
31. Dence CW (1971) in *Lignins. Occurrence, Formation, Structure and Reactions*, eds Sarkanen KV, Ludwig CH (Wiley Interscience, New York), pp 373–432.
32. Lapiere C (1991) in *Forage Cell Wall Structure and Digestibility*, eds Jung HG, Buxton DR, Hatfield RD, Ralph J (Am Soc Agron, Madison, WI), pp 133–166.
33. Simons BH, Barnett P, Vollenbroek EGM, Dekker HL, Muijsers AO, Messerschmidt A, Wever R (1995) *Eur J Biochem* 229:566–574.
34. Rogel JA, Ariza FA, Silla RO (2000) *Wetlands* 20:357–372.
35. Au DWT, Jones EBG, Vrijmoed LLP (1999) *Mycol Res* 103:865–872.
36. Bergbauer M, Newell SY (1992) *FEMS Microbiol Ecol* 86:341–347.
37. Inderbitzin P, Kohlmeyer J, Volkmann-Kohlmeyer B, Berbee ML (2002) *Mycologia* 94:651–659.
38. de Jong E, Field JA, Spinnler HE, Wijnberg J, de Bont JAM (1994) *Appl Environ Microbiol* 60:264–270.
39. Verhagen FJM, Swarts HJ, Wijnberg J, Field JA (1998) *Chemosphere* 37:2091–2104.
40. Pellinen J, Joyce TW, Chang H-M (1988) *Tappi J* 71:191–194.
41. Fetzner S, Lingens F (1994) *Microbiol Rev* 58:641–685.
42. Huynh VB, Chang H-M, Joyce TW, Kirk TK (1985) *Tappi J* 68:98–102.
43. Mohn WW, Tiedje JM (1992) *Microbiol Rev* 56:482–507.